SUPPLEMENTARY DATA

Methods

Construction and purification of eIF5B mutants

The human eIF5B<sub>587-1220</sub> coding region was subcloned by PCR between BamHI/HindIII restriction sites of pET28a. The resulting pET28a-ΔeIF5B<sub>587-1220</sub> vector was used to generate plasmids by PCR for expression of His<sub>6</sub>- and T7-Tag containing ΔeIF5B<sub>587-1220</sub> cysteine mutants. First, C853A, C1092A, C1126A and C1158A substitutions were combined to obtain a partially cysteine-less mutant, pcl-ΔeIF5B<sub>587-1220</sub>. Cysteine residues were introduced individually at residues 627, 766, 799, 810, 847 (G Domain), 853, 865, 869, 884, 887, 894, 898, 918, 936, 945, 952, 961, 968 (Domain 2), 1002, 1028, 1042, 1052, 1058, 1064 (Domain 3), and 1129, 1137, 1147, 1162, 1178, 1188, 1189, 1215 (Domain 4) of pcl-ΔeIF5B<sub>587-1220</sub> to create ΔeIF5B<sub>587-1220</sub> mutants with surface-exposed cysteines. Recombinant proteins were expressed in E. coli BL21(DE3) grown in 4 liters of LB at 37°C to an OD<sub>600nm</sub> of 0.2-0.3 followed by induction with 1 mM IPTG and 2 hours’ further growth at 30°C. After sonication, soluble fractions of ΔeIF5B<sub>587-1220</sub> mutants were purified on Ni<sup>2+</sup>-NTA (QIAGEN), followed by anion-exchange chromatography on a MonoQ column from which they were eluted with a 30-500 mM KCl gradient at ~100 mM KCl. Purified proteins were concentrated on YM-30 microcons (Millipore) and transferred to H300 buffer (80 mM HEPES (pH 7.5), 300 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10% glycerol). The yield of soluble ΔeIF5B<sub>587-1220</sub> mutants was very low (100-600 µg of protein per 4 liters of culture).

Purification of initiation factors, ribosomal subunits and aminoacylation of initiator tRNA

Native eIFs 2, 3, 5B and ribosomal subunits were purified from rabbit reticulocyte lysate, and recombinant eIFs 1, 1A and 5 were expressed in E. coli and purified as described (Pestova et al., 1996; Pestova et al., 1998a, b, Pestova et al., 2000). For hydroxyl radical cleavage experiments, ribosomal subunits were transferred into H100 buffer (80 mM HEPES, pH 7.5, 100 mM KCl, 5%
Glycerol, 2.5 mM MgCl₂) on the Y-30 Centricons (Millipore). In vitro transcribed tRNAᵢ_Met (Pestova and Hellen, 2001) was aminoacylated (spec. act. 30,000 cpm/pmol) using recombinant E.coli methionyl tRNA synthetase and [³⁵S]Methionine as described (Lomakin et al., 2006). tRNAᵢ_Met was phosphorylated (spec. act. 100,000 cpm/ pmol) using [³²P]-γATP and T4-Polynucleotide kinase.

Fe(II)-BABE modification of eIFΔ5B proteins

ΔeIF5B₅₈₇₋₁₂₂₀ mutant proteins were derivatized with Fe(II)-BABE as described (Culver and Noller, 2000; Lomakin et al., 2003) by incubating 80 µg of each ΔeIF5B₅₈₇₋₁₂₂₀ mutant with 1 mM Fe(II)-BABE in 100 µl H300 buffer for 30 min at 37°C. Derivatized proteins were separated from unincorporated reagent by buffer exchange on YM-30 microcons and stored at –80°C at 10-20 pmol/µl.

eIF5B GTPase assays

5 pmol of unmodified or Fe(II)-BABE derivatized wt or mutant ΔeIF5B₅₈₇₋₁₂₂₀ and 2.5 pmol 80S ribosomes were incubated in 20 µl buffer (20 mM Tris pH 7.5, 100 mM KCl, 2.5 mM MgCl₂) with 10 µM GTP and 2 µCi [³²P]-γGTP (5000Ci/mmol) for 30 min at 37°C. Aliquots (2 µl) were spotted onto polyethylenemine-cellulose plates for chromatography in 0.8 M LiCl/0.8 M acetic acid. GTPase activity of eIF5B was assayed by formation of [³²P]Pᵢ.

Ribosomal subunit joining and methionyl-puromycin synthesis assays

First, 48S complexes were assembled by incubating multiples of 50 µl reaction mixtures containing 3 pmol 40S subunits, 8 pmol eIF2, 3 pmol eIF3, 15 pmol eIFs 1 and 1A, 4 pmol [³⁵S]-Met-tRNAᵢ_Met (30,000 cpm/pmol) or 5'[^³²P]-Met-tRNAᵢ_Met (100,000 cpm/pmol) and 10 pmol of 35nt-AUG-32nt mRNA (Unbehaun et al., 2004) in buffer (20 mM Tris pH 7.5, 100 mM KAc,
0.25 mM spermidine, 2.5 mM MgCl$_2$ and 0.5 mM GTP) for 10 min at 37°C. To assay [$^{35}$S]-methionyl-puromycin formation as described (Pestova et al., 2000), 48S complexes assembled with [$^{35}$S]-Met-tRNA$_i^{\text{Met}}$ were further incubated with 2 mM puromycin, 4.5 pmol 60S subunits, 10 pmol eIF5 and 10 pmol native eIF5B, or unmodified or Fe(II)-BABE derivatized wt or mutant ΔeIF5B$_{587-1220}$ in the presence of 4 mM MgCl$_2$ for 30 min at 37°C. For the ribosomal subunit joining assay, 6 pmol 48S complexes assembled with [$^{32}$P]-Met-tRNA$_i^{\text{Met}}$ were further incubated with 7 pmol 60S subunits, 10 pmol eIF5 and 15 pmol native eIF5B, or wt or mutant ΔeIF5B$_{587-1220}$ in the presence of 4 mM MgCl$_2$ for 10 min at 37°C. Ribosomal complexes were separated by centrifugation through 10%–30% sucrose density gradients in a SW55 rotor at 50,000 rpm for 95 min, and association of [$^{32}$P]-Met-tRNA$_i^{\text{Met}}$ with 80S ribosomes was assayed by Cherenkov counting.

**Directed hydroxyl radical cleavage**

For hydroxyl radical cleavage of 18S/28S rRNA, binary 80S/[Fe(II)-BABE]-ΔeIF5B$_{587-1220}$ complexes were assembled by incubating 25 pmol 40S subunits, 25 pmol 60S subunits and 50 pmol [Fe(II)-BABE]-ΔeIF5B$_{587-1220}$ in 70 µl HRC buffer (80 mM HEPES at pH 7.5, 130 mM KCl, 5% glycerol, 3 mM magnesium acetate) containing 0.4 mM GTP-γS for 15 min at 37°C. Quaternary 80S/Met-tRNA$_i^{\text{Met}}$/mRNA/[Fe(II)-BABE]-ΔeIF5B$_{587-1220}$ complexes were formed by incubating 18 pmol 48S complexes (assembled with [$^{35}$S]-Met-tRNA$_i^{\text{Met}}$ or 5'-[$^{32}$P] Met-tRNA$_i^{\text{Met}}$ and purified by centrifugation through 10-30% sucrose density gradients) with 20 pmol 60S subunits, 50 pmol eIF5 and 50 pmol [Fe(II)-BABE]-ΔeIF5B$_{587-1220}$ in 70 µl HRC buffer with 0.4 mM GTP-γS for 15 min at 37°C. To generate hydroxyl radicals, reaction mixtures were supplemented with 0.025% H$_2$O$_2$ and 5 mM ascorbic acid (Culver and Noller, 2000) and incubated on ice for 10 min. Reactions were quenched by adding 20 mM thiourea. Ribosomal RNA and initiator tRNA were phenol-extracted, ethanol precipitated and analyzed by primer
extension using AMV RT and primers complementary either to different regions of 18S and 28S rRNA or to the 16 3’-terminal nucleotides of tRNA_{\text{Met}} (Lomakin et al., 2003). 5’[\text{32P}]-\text{Met}-tRNA_{\text{Met}} was analyzed directly by gel electrophoresis and autoradiography.

**Modeling**

The eIF5B/80S ribosome interaction was modeled using the crystal structure of the *E. coli* 70S ribosome (Schuwirth et al., 2005; PDB codes 2AW7 (small subunit) and 2AWB (large subunit)) and the crystal structure of *M. thermoautotrophicum* eIF5B (Roll-Mecak et al., 2000; PDB entry 1G7T) designated as "free eIF5B", as well as a model for eIF5B in which individual domains were oriented as in the Cryo-EM reconstruction of ribosome-bound IF2 (Allen et al., 2005; PDB code 1ZO1) designated as "modeled eIF5B". Docking was done interactively in MOLMOL (Koradi et al., 1996). The distances between C\_\beta atoms of eIF5B’s residues and RNA phosphate backbones of their corresponding targets were interpreted as <15 Å for strong cleavages and as <20 Å for weaker cleavages. Initially, docking was done for individual domains of eIF5B. Their resulting orientations were then compared with the structure of the intact eIF5B (both “free eIF5B” and “modeled eIF5B”), and the global fit was optimized to the cleavage data while minimally affecting the docking quality of the individual domains.

The file Model_eIF5B_ribosome.pdb contains the coordinates of the docking model for the eIF5B/ribosome complex. The file contains the crystal structure of the *E. coli* ribosome from PDB entries 2AW7.pdb (30S ribosomal subunit, molecules 1-22) and 2AWB.pdb (50S ribosomal subunit, molecules 23-54), and eIF5B (mol. 55), modeled as described in the text. The structures of the individual eIF5B domains in the model are from the X-ray structure of archaeal eIF5B (PDB entry 1G7T.pdb), while their mutual orientation is based on the interdomain orientation of ribosome-bound IF2 (PDB entry 1ZO1.pdb). A complete list of the molecules in the model can be found in the header of the file Model_eIF5B_ribosome.pdb.
Results

Mapping conserved residues on eIF5B’s structure (Supplementary Fig. 5) indicated that most conserved surfaces are involved in ribosomal interactions. The only large universally conserved surface corresponds to the GTP-binding region of the G domain (including G1-G4 motifs, Switch 1 and Switch 2), which contacts H95, probably also rpL23 and possibly H91. A universally conserved (but not 100% identical) surface of domain 4 contacts H69 and the H67-H71 linker. The surface of domain 2 that contacts 18S rRNA and the surfaces of domain 3 that contact rpS23 and rpL23 are conserved within each of the three kingdoms, but not necessarily between them. The surface of domain 3, that, according to our cleavage data, could contact the GAC, is also universally conserved.
REFERENCES


Pestova TV, Lomakin IB, Lee JH, Choi SK, Dever TE, Hellen CUT (2000b) The joining of
ribosomal subunits in eukaryotes requires eIF5B. *Nature* **403**: 332–335


Figure 1. GTPase activity of eIF5B cysteine mutants. (A) Thin-layer chromatography analysis of the stimulation of the GTPase activity of ΔeIF5B<sub>587-1220</sub> cysteine mutants by 80S ribosomes. Positions of [<sup>32</sup>P]GTP and [<sup>32</sup>P]Pi are indicated. (B) Time courses of methionylpuromycin synthesis by 80S ribosomes assembled from 48S complexes (formed on 35nt-AUG-32nt mRNA with [<sup>35</sup>S]Met-tRNA<sub>Met</sub>), 60S subunits, eIF5, ΔeIF5B<sub>587-1220</sub> in the presence of GTP, GMPPNP and GTP-γS, as indicated.

Figure 2. Directed hydroxyl radical cleavage of 18S and 28S rRNA in 80S/Met-tRNA<sub>Met</sub>/mRNA/[Fe(II)-BABE]-ΔeIF5B<sub>587-1220</sub> ribosomal complexes from Fe(II) tethered to cysteines on the surface of eIF5B domain 1 (A) and domain 2 (B-E). Hydroxyl radicals were generated in 80S/Met-tRNA<sub>Met</sub>/mRNA/[Fe(II)-BABE]-ΔeIF5B<sub>587-1220</sub> quaternary complexes from Fe(II) tethered to surface positions on eIF5B, as indicated; positions from which hydroxyl radicals cleaved 18S/28S rRNA are shown in bold. Control reactions in lanes marked “pcl-ΔeIF5B” were carried out in the presence of partially cysteine-less eIF5B. Sites of hydroxyl radical cleavage were mapped by primer extension inhibition. The positions of cleaved nucleotides are shown on the right. Reference lanes G, A, T and C depict rRNA sequence generated from the same primer.

Figure 3. Directed hydroxyl radical cleavage of 18S and 28S rRNA in 80S/Met-tRNA<sub>Met</sub>/mRNA/[Fe(II)-BABE]-ΔeIF5B<sub>587-1220</sub> ribosomal complexes from Fe(II) tethered to cysteines on the surface of eIF5B domain 3 (A-G) and domain 4 (H). Hydroxyl radicals were generated in 80S/Met-tRNA<sub>Met</sub>/mRNA/[Fe(II)-BABE]-ΔeIF5B<sub>587-1220</sub> quaternary complexes from Fe(II) tethered to surface positions on eIF5B, as indicated; positions from which hydroxyl
radicals cleaved 18S/28S rRNA are shown in bold. Control reactions were done using partially cysteine-less eIF5B (lanes marked “pcl-ΔeIF5B”). Sites of hydroxyl radical cleavage were mapped by primer extension inhibition. The positions of cleaved nucleotides are shown on the right. Reference lanes G, A, T and C depict rRNA sequence generated from the same primer.

**Figure 4. Directed hydroxyl radical probing of initiator tRNA in 80S/Met-tRNA\textsuperscript{Met}/mRNA/[Fe(II)-BABE]-ΔeIF5B\textsubscript{587-1220} complexes from Fe(II) tethered to cysteine residues on the surface of eIF5B domain 4.**

Hydroxyl radicals were generated in 80S/Met-tRNA\textsuperscript{Met}/mRNA/[Fe(II)-BABE]-DeIF5B\textsubscript{587-1220} quaternary complexes from Fe(II) tethered to surface positions on eIF5B domain 4, as indicated. (A) Autoradiograph of the gel showing analysis of cleavage of 5’ end-labeled tRNA\textsuperscript{Met}. (B) Primer extension analysis of cleavage of tRNA\textsuperscript{Met} using a primer complementary to the 16 3’-terminal nucleotides of tRNA. Control reaction mixtures either did not contain ΔeIF5B or contained the partially cysteine-less ΔeIF5B mutant (“no eIF5B” and “pcl-ΔeIF5B” lanes, respectively). Reference lanes C, T, A and G depict mammalian tRNA\textsuperscript{Met} sequence generated using the same primer complementary to the 16 3’-terminal nucleotides of tRNA. Positions of tRNA\textsuperscript{Met} nucleotides are shown to the right of each panel.

**Figure 5. The positions of the conserved surfaces of eIF5B relative to rRNA in the modeled eIF5B/80S(70S) ribosome complex.** eIF5B is shown in surface representation (gray). The surfaces of eIF5B that are 80% identical in all kingdoms are colored red, those that have 80% similarity in all kingdoms are orange, and those that have 80% similarity inside each kingdom, but not necessarily between kingdoms, are blue. 16S and 23S rRNAs in the *E. coli* 70S ribosome crystal structure (Schuwirth et al, 2005) are shown as light-yellow and light-gray ribbons, respectively.
Unbehaun et al., Supplementary Figure 1
A Domain 1 of eIF5B
Helix 95 of 28S rRNA

B Domain 2 of eIF5B
helices 4 and 15 of 18S rRNA

C Domain 2 of eIF5B
helix 17 of 18S rRNA

D Domain 2 of eIF5B
helices 5 and 15 of 18S rRNA

E Domain 2 of eIF5B
helices 3, 5 and 6 of 18S rRNA

Unbehau et al., Supplementary Figure 2
Unbehaun et al., Supplementary Figure 3

A. Domain 3 of eIF5B
   Helix 95 of 28S rRNA

B. Domain 3 of eIF5B
   Helix 91 of 28S rRNA

C. Domain 3 of eIF5B
   Helix 89 of 28S rRNA

D. Domain 3 of eIF5B
   Helix 71 of 28S rRNA

E. Domain 3 of eIF5B
   Helix 69 of 28S rRNA

F. Domain 3 of eIF5B
   Helices 43 and 44 of 28S rRNA

G. Domain 3 of eIF5B
   Helix 44 of 18S rRNA

H. Domain 4 of eIF5B
   Loop connecting Helices 67 and 71 of 28S rRNA
Unbehaun et al., Supplementary Figure 4
Unbehau et al, Supplementary Figure 5